An autoregulatory feedback loop directs the localized expression of the 
*Drosophila* CPEB protein Orb in the developing oocyte

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SUMMARY

The RRM-type RNA binding protein Orb plays a central role in the establishment of polarity in the *Drosophila* egg and embryo. In addition to its role in the formation and initial differentiation of the egg chamber, *orb* is required later in oogenesis for the determination of the dorsoventral (DV) and anteroposterior (AP) axes. In DV axis formation, Orb protein is required to localize and translate *gurken* mRNA at the dorsoanterior part of the oocyte. In AP axis formation, Orb is required for the translation of *oskar* mRNA. In each case, Orb protein is already localized at the appropriate sites within the oocyte before the arrival of the mRNAs encoding axis determinants. We present evidence that an autoregulatory mechanism is responsible for directing the on site accumulation of Orb protein in the *Drosophila* oocyte. This *orb* autoregulatory activity ensures the accumulation of high levels of Orb protein at sites in the oocyte that contain localized *orb* message.

Key words: Orb, Oogenesis, Autoregulation, Translation, *Drosophila*
orb\textsuperscript{mel} is not fully expressed and almost 20% of the embryos hatch as larvae. The morphological abnormalities in orb\textsuperscript{mel} egg shells and embryos can be directly attributed to an improper execution of the mRNA localization pathways responsible for axes determination during oogenesis.

How orb functions in mRNA localization is best understood in the posterior pathway. Christerson and McKearin (Christerson and McKearin, 1994) found that osk mRNA is transported to the posterior end of the oocyte in orb\textsuperscript{mel} chambers, but does not seem to be properly anchored to the pole. This RNA localization defect is likely due to a failure in the translation of osk message when it arrives at the pole. This possibility was first suggested by the discovery that Orb is homologous to the Cytoplasmic Polyadenylation Element Binding protein (CPEB) found in maturing Xenopus leavis eggs (Hake and Richter, 1994; Stebbins-Boaz et al., 1996). CPEB is required to activate translation of masked maternal mRNAs. orb seems to have an analogous function in the fly posterior pathway, activating the translation of osk mRNA when it arrives at the pole (Chang et al., 1999). When Orb is absent, Osk protein is not synthesized, and osk message diffuse away. orb appears to play a similar role in DV polarity, in this case controlling the translation of both K(10) and grk mRNA (Neuman-Silberberg and Schupbach, 1996; unpublished data).

In each of these polarity pathways, Orb protein is already on site before the mRNAs encoding the axes determinants arrive. In fact, Orb is one of the first proteins localized to the oocyte (Lantz et al., 1994). In the germarium, it can be detected in the oocyte as soon as the 16-cell cyst is formed. From this stage through the remaining previtellogenic stages (stages 1-7) Orb accumulates at the posterior of the oocyte forming a graded cap that extends anteriorly along the cortex. By the time osk mRNA is transported to the posterior pole at stages 8-9, high levels of Orb are already present. Similarly, when the oocyte nucleus moves to the dorsoanterior part of the oocyte, there is a rapid accumulation of Orb protein along the entire oocyte cortex. As a result Orb is already present at the oocyte-nurse cell margin when K(10) and Squid localize grk mRNA around the oocyte nucleus.

If Orb protein localization precedes and is independent of the two polarity pathways, what directs its distribution? The most likely mechanism is an mRNA localization pathway that targets orb mRNA to specific regions of the oocyte and then activates translation. orb mRNA exhibits a dynamic pattern of accumulation during oogenesis that closely parallels if not anticipates the protein distribution (Lantz et al., 1992, Lantz et al., 1994). In pre-vitellogenic stages, orb mRNA accumulates in a cap at the posterior pole while at the onset of vitellogenesis, this cap disappears and the message shifts to the anterior margin. While an mRNA localization pathway is likely to direct Orb accumulation in the oocyte, very little is known about this pathway. The long 3' UTR of the female orb mRNA has been shown to contain cis-acting localization elements (Lantz and Schiedl, 1994), however, the trans-acting factors that bind to the message, target it to different regions of the oocyte and control translation remain to be elucidated. We have investigated the role of Orb protein in the orb mRNA localization pathway. We show that Orb protein autoregulates its own expression through sequences in the 3' UTR of the orb mRNA. This autoregulatory activity provides a mechanism for ensuring the on site accumulation of high levels of Orb protein.

**MATERIALS AND METHODS**

**Fly Stocks**

The w\textsuperscript{1160} is described by Lindsley and Zimm (Lindsley and Zimm, 1992). orb\textsuperscript{443} is from a collection of female steriles on the third chromosome (a gift from C. Nusslein-Volhard) and is described by Lantz et al. (Lantz et al., 1994). orb\textsuperscript{mel} is described by Christerson and McKearin (Christerson and McKearin, 1994). The hsp\textsuperscript{83} lacZ transgenes containing DNA sequences from the orb 3' UTR have been described in detail by Lantz and Schiedl (Lantz and Schiedl, 1994). The hsp\textsuperscript{83}:lacZ reporter transgene with the male specific lethal 2 (msl2) 3' UTR instead of the orb 3' UTR was a gift from J. Yanowitz.

**Immunoprecipitation**

Ovaries of well-fed 2- to 3-day old wild-type females (30) were dissected in 1x PBS and frozen immediately on dry ice. Ovary extract was prepared by adding 100 μl of ice-cold IP buffer (20 mM Hapes, pH 7.5, 150 mM NaCl, 2.5 mM MgCl\textsubscript{2}, 250 mM sucrose, 0.05% NP40, 0.5% Triton X-100, 1 mM PMSF, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 1 μg/ml leupeptin) and homogenized with a plastic pestle in a 1.5 ml microfuge tube with 3-4 freeze/thaw cycles. The homogenate was filtered through glass wool in the end of a 200 μl pipette tip and centrifuged at 3000 rpm for 5 minutes. The supernatant was removed and mixed with 40 units of RNasin, 30 μl of antibody-coupled Protein-A beads (Bio-Rad) for 2 hours to overnight at 4°C on a rotator. The IP mix was centrifuged at low speed in the cold room to remove the supernatant. The Protein-A beads were then washed 4-5 times with 20 volumes of IP buffer without MgCl\textsubscript{2}. 5-10 μl of the protein-A beads were analyzed on a western blot for the presence of Orb protein by detection with enhanced chemiluminescence system from Amersham. The remainder of the beads were phenol/chloroform extracted and precipitated with glycogen to isolate mRNAs. The RT-PCR assay was performed as described below.

**PCR Poly(A) assay**

Total ovary RNA and the mRNAs from IP with anti-Orb and anti-Dorsal antibodies were reverse transcribed using an anchored oligo(dT) primer (as in Salles et al., 1994). PCR was performed using the anchor primer paired with a gene-specific primer for the orb 3' UTR: 5'-CATAGCAAGCCCGCGACTCG-3', starting at base no. 4128 of the published sequence in Lantz et al. (Lantz et al., 1992), for nos 3' UTR: 5'-ACTTGTGTAATCGTGTTGGCGG-3' starting approximately at base no. 2387 of the sequence published by Wang and Lehmann (Wang and Lehmann, 1991) and for bed 3' UTR: 5'-CTAGTACGAGCGCGCAGCG-3', starting at base no. 4172 of the sequence published by Berleth et al. (Berleth et al., 1988) as follows: 1 cycle of 4 minutes at 94°C, 25 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C, followed by 1 cycle for 15 minutes at 72°C. The PCR products were analyzed on several different gel systems (non-denaturing agarose gels with TBE buffer; alkaline agarose gels with 10 mM EDTA, 50 mM NaOH; 1.2% vertical formaldehyde agarose gels in MOPs buffer) and transferred to nitrocellulose or Zeta Probe membrane (from Bio-Rad) for 3-4 hours to overnight at 4°C. The blots were hybridized with random primed probes made from orb, nos and bed 3' UTR sequences cloned in BS plasmid vectors.

**UV cross-linking**

UV cross-linking experiments were preformed as described by Chang et al. (Chang et al., 1999) with only minor modifications; the reaction buffer contained two divalent cations. In addition to 1.5 mM MgCl\textsubscript{2}, 0.2 mM ZnCl\textsubscript{2} was included.

**Immunocytochemistry and western analysis**

Ovaries were dissected in PBS, fixed and stained for Orb protein as described previously (Lantz et al., 1994). Alexa-546 (Molecular Probes, Inc.) -conjugated anti-mouse antibody was used with mouse anti-Orb and mouse anti-β-galactosidase antibodies. Yo-Pro was used
for staining the nuclei. Imaging was done by laser scanning confocal microscopy (Krypton-Argon Laser, Bio-Rad MRC 600). Western analysis was done as described previously (Lantz et al., 1994).

RNA in situ hybridization on whole-mount ovaries

Ovaries were dissected in cold 1× PBS, teased apart, fixed and hybridized as described by Tautz and Pfeifle (Tautz and Pfeifle, 1989) and modified by Suter and Steward (Suter and Steward, 1991). Digoxigenin-labeled antisense RNA probes were synthesized by use of the RNA genius kit (Boehringer Mannheim). Ovaries were mounted in Aqua-polymount (Polysciences, Inc.).

RESULTS

Antisense RNA complementary to the orb 3’ UTR interferes with orb gene function

Previous studies have shown that orb mRNA localization depends upon sequences in the approx. 1 kb 3’ UTR of the female transcript (Lantz and Schedl, 1994). When a tagged orb female cDNA is ectopically expressed in the germline, the tagged transcript displays a localization pattern like that of the endogenous orb message. Removal of the 3’ UTR sequences from the tagged orb cDNA prevents the localization. Conversely, the localization pattern of the endogenous message can be recapitulated by fusing the UTR to a heterologous transcript encoding E. coli β-galactosidase. Deletion mapping pinpoints the localization elements to an approx. 300 nucleotide sequence within the UTR.

While these findings indicate that the 3’ UTR is required to localize orb mRNA, they do not reveal whether localization is important for orb activity. Since attempts to generate orb cDNA rescue constructs have been unsuccessful, we decided to address this question by testing whether antisense RNAs complementary to sequences in the female orb 3’ UTR interfere with orb gene function. We reasoned that the antisense RNAs would hybridize to the 3’ UTR of the endogenous orb message, preventing components of the localization pathway from recognizing their target sites.

To assess the feasibility of this strategy, we asked whether an hsp83 construct, NH, which expresses β-gal coding sequences fused to a near full length (815 bp) antisense orb 3’ UTR (plus a sense SV40 polyadenylation signal; Fig. 1), has any phenotypic consequences. As shown for two different NH transgenic lines in Table 1A, between 3 and 6% of the eggs produced by transgenic females had ventralized chorions (Fig. 1), while the frequency of similar defects in eggs from the wild-type control was less than 0.5% (Table 1A).

The ventralized chorions are indicative of a failure in the grk-top signaling pathway. Since precisely the same phenotype is found in eggs from females homozygous for the weak orb‡‡ allele (Christerson and McKearin, 1994), a plausible hypothesis is that the antisense RNA interferes with the functioning of the endogenous orb gene. In this case the frequency of chorion defects should be increased by reducing the orb gene dose. orb is weakly haplo-insufficient for its function in the grk-top pathway and females heterozygous for the null allele orb‡‡ produce a significant number of eggs which have ventralized chorions. The frequency of chorion defects in eggs from orb‡‡/+ mothers is 10-15%. As shown in Table 1A, a single copy of the NH transgene in orb‡‡/+ females increases the frequency of chorion defects to over 20% for the NH4B1 line and to 45% for the NH24C line. These findings indicate that the antisense NH transgene acts as a dominant negative.

Transgenes expressing the orb 3’ UTR in the sense orientation also interfere with orb gene function

As a control for the antisense NH transgene, we examined eggs produced by females carrying a second transgene, HD, which ectopically expresses lacZ coding sequences fused to the female orb 3’ UTR in a sense, rather than an antisense orientation (Fig. 1; Lantz and Schedl, 1994). The UTR sequences in HD are sufficient to recapitulate the complex localization pattern of the endogenous orb mRNA. (HD is an orb genomic fragment and also contains sequences downstream of the female orb polyadenylation site.)

Since transcripts from the sense transgene should not hybridize to orb mRNA, we expected that they would have no effect on orb gene function. However, contrary to this expectation, the sense 3’ UTR transgene induced chorion defects at a frequency similar to that seen for the antisense transgene. Nearly 5% of the eggs have ventralized chorions when the HD transgene is present in a single copy, while about 11% have a ventralized chorion when there are two copies (Table 1B).

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**Table 1. Egg shell phenotypes induced by different transgenes**

<table>
<thead>
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<th>Genotype</th>
<th>Total</th>
<th>DV defects*</th>
<th>% DV defects*</th>
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<tr>
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<td>45.0</td>
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<td>22.0</td>
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</table>

*Frequency of ventralized egg shells.

w/w is the parental stock for isolating transgenic lines for the different lacZ reporter constructs. These constructs have a mini-white marker.

NH4B1 and NH24C1 are two independent transgenic lines for the NH construct (see Fig. 1A). Both are inserted on the 3rd chromosome.

NH4B1/NH4B1 are females that are homozygous for the NH4B1 insert; NH4B1 orb‡‡ is a recombinant between the transgene and the orb‡‡ allele. In this experiment the recombinant chromosome is over the TM3 balancer.

HD19G is a transgenic line for the HD construct (see Fig. 1A) which is inserted on the third chromosome. Other genotypes are as for NH4B1. Two transgenes, AN and AE, carrying sub-fragments from the orb 3’ UTR (see Fig. 1A) were also tested either as homozygotes or when combined with orb‡‡.

Note that AN61D is inserted on the 2nd chromosome.

Total: number of eggs scored; DV defects: the number of eggs exhibiting a ventralized egg shell phenotype; %DV defects: the percentage of eggs exhibiting ventralized egg shells.
Sequences conferring dominant negative activity overlap with the orb mRNA localization signal

Since the sense HD transgene contains the female orb 3'UTR plus downstream genomic sequences, the formal possibility exists that the non-coding genomic sequence and not the orb 3'UTR is responsible for the dominant negative activity. To exclude this possibility, we tested transgenes in which fragments from the orb 3'UTR are inserted between the lacZ protein coding sequences and an SV40 polyadenylation sequence. As previous studies showed that sequences near the middle of the UTR are sufficient to localize lacZ RNA in a pattern like the endogenous message (Lantz and Schedl, 1994), we examined two transgenes, AN and AE, which have UTR sequences spanning this critical region (Fig. 1). AN has a 570 nt UTR while the AE transgene has a 364 nt UTR.

The phenotypic effects of the AN transgene are comparable to the original hsp83: lacZ orb 3'UTR transgene (Table 1C). When present in two copies in a wild-type background, about 10% of the eggs produced by AN61D mothers have ventralized chorions. When a single copy of the AN transgene is introduced into females heterozygous for orb^{+43}, about 50% of the eggs are defective. While the phenotypic effects of the AN61D line are stronger than the HD transgene, position effects might account for this difference as a second AN line is weaker (not shown). The smaller AE transgene also disrupts DV polarity (see Table 1C). These findings demonstrate that sequences from the orb 3'UTR are responsible for the dominant negative activity of the orb sense transgenes and suggest that elements conferring this activity overlap with those involved in localizing orb mRNA during oogenesis.

β-galactosidase expression from the orb 3'UTR transgene depends upon orb gene function

We suppose that both the sense and antisense transgenes act as dominant negatives because they interfere with the proper expression of Orb protein; however, since RNA from the sense transgenes cannot hybridize to orb message, the mechanism must be different from that of the antisense transgene. One plausible hypothesis is that the sense lacZ orb 3'UTR transcript competes with the endogenous orb mRNA for some limiting factor that is essential for Orb protein expression. While a number of factors could be limiting, the fact that orb is haploinsufficient makes Orb itself a good candidate. This would imply that Orb is required to promote the translation of orb mRNA. In this case the dominant negative activity of the lacZ orb 3'UTR mRNA would arise from a competition with endogenous orb mRNA for Orb function. When Orb acts on the lacZ orb 3'UTR mRNA instead of orb mRNA, it would promote β-galactosidase not Orb expression. As a consequence, Orb protein production would be downregulated by the transgene mRNA.

If this autoregulatory hypothesis is correct, then the expression of β-galactosidase from the lacZ orb 3'UTR transgenes should depend on orb function. To determine if this is the case we compared the level of β-galactosidase expressed from the HD transgene in orb^{+43}/+ females with orb^{+43}/orb^{mel} females. As in orb^{mel} homozygotes, orb activity in the 343/mel mutant combination appears comparatively normal in previtellogenic stages; however, by the onset of vitellogenesis defects in orb dependent mRNA localization pathways become evident (Christerson and McKearin, 1994; Chang et al., 1999).
levels of β-galactosidase are seen in nuclei. There appears to be a very slight but consistent decrease in the level of β-galactosidase in vitellogenic orb343/+ egg chambers, while the level of protein in earlier chambers is generally close to that seen in a wild-type background. The most striking reduction in β-galactosidase expression is seen in orb343/orbmel ovaries. As expected from the defects in orb function evident in vitellogenic orb343/orbmel chambers, very little β-galactosidase is observed after stage 7. Moreover, even in earlier stages the level of β-galactosidase is less than in wild type. These findings indicate that the expression of β-galactosidase from the lacZ orb 3′ UTR transgene, particularly in vitellogenic stages, requires wild type orb gene function.

Orb protein expression is inhibited by the orb 3′ UTR transgene

A second prediction of the autoregulatory model is that mRNA produced by the lacZ orb 3′ UTR transgenes should down-regulate translation of the endogenous orb mRNA by competing for orb activity. To test this prediction, we sought to maximize the dominant negative effects of the HD transgene. In wild-type females the frequency of DV defects induced by even two copies of the transgene is only a few percent (see Table 1). Consequently, we did not expect, nor did we observe, transgene-induced changes in Orb expression in a wild-type background (data not shown). However, if there is only a single functional orb gene, the frequency of D-V defects in the presence of two copies of the HD transgene can be more than 50%. Under these conditions clear cut changes in Orb expression could be observed.

The level and distribution of Orb protein in previtellogenic

As a control for a general reduction in protein synthesis or a change in β-galactosidase stability in the absence of wild-type orb function, we introduced a lacZ transgene which has a 3′ UTR from the msl-2 mRNA into the same genetic backgrounds.

Western blots of ovaries from orb343/+ and orb343/orbmel females carrying either the orb or msl-2 transgenes were probed with antibody directed against β-galactosidase and, as a loading control, the snRNP protein Snf (Fig. 2). Loss of orb function has no effect on β-galactosidase expression from the msl-2 3′ UTR transgene; the level of β-galactosidase is the same in ovaries lacking a wild-type orb gene as in ovaries that have a wild-type copy. (Similar results were obtained for another lacZ control, the hsp83: lacZ SV40 3′ UTR transgene; not shown.) In contrast, β-galactosidase expression from the HD transgene depends upon orb and the level of β-galactosidase is substantially reduced in ovaries from females lacking a wild-type gene (compare 343P/+ with 343P/mel).

To confirm and extend these findings we examined β-galactosidase expression from the HD lacZ orb 3′ UTR transgene in wild-type and orb mutant backgrounds using confocal microscopy. In wild-type ovaries, β-galactosidase from the lacZ orb 3′ UTR transgene can be detected throughout much of oogenesis (Fig. 3). Though cytoplasmic protein is observed in wild-type chambers, the highest
stages of orb343/+ ovaries appears to be similar either with or without the transgene (Fig. 4A,D). However, after the onset of vitellogenesis, the accumulation of Orb protein in chambers from transgene ovaries is reduced compared to chambers from ovaries lacking the transgene (compare the orb343/P/+ chambers in the middle and right panels of Fig. 4). We also examined ovaries from orb343/+ females that had a single copy of the lacZ orb3 3′ UTR transgene. Although vitellogenic chambers with abnormally low levels of Orb protein were observed, the Orb staining pattern in most vitellogenic chambers was similar to that of the orb343/+ control without transgene. Presumably this reflects the fact that the frequency of eggs with DV defects is lower when orb343/+ females have only a single copy of the transgene then when they have two copies. Based on these findings and the results described in the previous sections, we conclude that Orb protein positively autoregulates its own expression, most probably by activating the translation of orb mRNA through sequences in the 3′ UTR.

Fig. 4. The hsp83 LacZ-orb3 3′UTR transgene interferes with orb protein expression. Expression of Orb protein in ovaries from orb343/+ and orb343 P/P females; (A,D) previtellogenic chambers; (B,C,E,F) vitellogenic chambers. Staining in red is Orb protein while nuclei are stained in blue. In orb343/+ ovaries, the pattern of accumulation is similar to that observed in wild-type ovaries (data not shown; see Lantz et al., 1994). When two copies of the lacZ orb 3′ UTR transgene are present, the expression of Orb protein in orb343/+ ovaries is reduced. The effects of the transgene on Orb protein accumulation are most evident after stages 6-7.

orb function is required for orb mRNA localization
Since Orb protein accumulates at sites containing orb mRNA, we reasoned that orb function might be required not only for translation but also for orb mRNA localization. In wild type ovaries, orb mRNA can first be detected in the germarium where it preferentially accumulates in the presumptive oocyte of newly formed 16 cell cysts (Lantz et al., 1992 and orb343 P/+ ovaries in Fig. 5A). Between stages 1 and 7, the highest concentrations of mRNA are found in a cap at the posterior of the oocyte. This posterior cap disappears at the onset of vitellogenesis, and most orb mRNA is found in a ring along the oocyte-nurse cell border (Lantz et al., 1992 and Fig. 5A).

To determine if Orb plays a role in localizing its own mRNA, we examined the distribution of orb message in orb343/forbmel ovaries either with (Fig. 5A) or without (not shown) the HD transgene. Orb protein expression in the germarium and in stages 1-6 egg chambers of mutant ovaries

Fig. 5. orb mRNA localization requires orb gene function. (A) Four examples of orb mRNA localization in orb343 P/+ and orb343 P/orbmel ovaries. Ovaries were hybridized with an antisense RNA probe synthesized from an orb cDNA. The localization of orb mRNA in the orb343 P/+ ovaries is similar to that previously described for wild-type ovaries (Lantz et al., 1992; data not shown). In the orb mutant combination, orb343 P/orbmel, orb mRNA localization in early stages is not discernibly altered; however, from stage 5-6 onward, defects in the pattern of the orb mRNA localization are evident. Not shown here is the localization pattern of orb mRNA in ovaries from orb343/+ and orb343 P/orbmel females that do not carry the lacZ orb 3′ UTR transgene. As indicated in the text, similar results were obtained. (B) lacZ orb 3′ UTR mRNA localization in orb343 P/+ and orb343 P/orbmel ovaries. In this experiment whole mounts of orb343 P/+ and orb343 P/orbmel ovaries were hybridized with an antisense RNA probe synthesized from a lacZ clone. The localization of lacZ orb 3′ UTR mRNA in the orb343 P/+ ovaries is the same as that previously described in wild-type transgenic ovaries (Lantz and Schedl, 1994; data not shown). In the orb mutant combination, orb343 P/orbmel, the localization pattern of lacZ orb 3′ UTR mRNA in early stages is the same as that in orb343 P/+ ovaries; however, from stage 5-6 onward, abnormalities are evident. Arrows indicate hybridization (to orb or lacZ mRNA) along the anterior margin of the oocyte.
resembles wild type; however, defects in Orb protein accumulation can be detected around stage 7 (data not shown; Christerson and McKearin, 1994; Chang et al., 1999). Many vitellogenic chambers lack the Orb protein cap at the posterior, and there is a marked reduction in the amount of protein associated with the oocyte cortex. The orb mRNA localization pattern in the germarium and early egg chambers of orb343/orbmel mutant ovaries is, like the protein, generally indistinguishable from the wild-type control. High concentrations of orb mRNA are found in the oocyte and the message accumulates in a posterior cap. However, beginning at stages 5-6 a range of abnormalities in orb mRNA localization are observed. In many stage 5-6 chambers, orb mRNA is distributed uniformly in the oocyte instead of being concentration at the posterior. These abnormalities in mRNA localization appear to precede the appearance of readily detectable defects in Orb protein accumulation which are usually first evident in the mutant ovaries slightly later. Even more severe defects in mRNA localization are found after the onset of vitellogenesis. Unlike wild-type vitellogenic chambers, there is typically no anterior ring of orb mRNA. Instead the message is present throughout the oocyte-nurse cell complex (see Fig. 5A). In some orb343/orbmel chambers, the overall level of orb mRNA also appears to be reduced. While the orb mRNA distribution in most vitellogenic chambers is aberrant, we occasionally observe chambers in which the localization pattern resembles wild type. (Similar results have been obtained by L. Christerson and D. McKearin, personal communication.)

Although these results demonstrate that the orb mRNA localization pathway is disrupted in orb343/orbmel ovaries, there is a potential complication. The failure to properly localize orb mRNA could be due to a defect in some key cis-acting element in the orb mRNAs produced by the mutant orb343 or orbmel genes, rather than to a lack of sufficient quantities of functional Orb protein. To address this problem, we examined the localization of mRNA expressed by the HD lacZ orb 3’ UTR transgene. The orb 3’ UTR sequences in the HD fragment are sufficient to direct the accumulation of lacZ mRNA in wild-type ovaries in a pattern that closely resembles that of the endogenous mRNA (Lantz and Schedl, 1994; see also the orb343/P+ ovaries in Fig. 5B). If Orb functions in the localization of this heterologous mRNA, then its localization pattern should be altered in the orb343/orbmel ovaries. As shown in Fig. 5B, this is the case. Like the endogenous orb message, mRNA expressed by the HD transgene is not properly localized after stages 5-6 in the mutant ovaries. Typically the HD mRNA is distributed throughout the egg chamber instead of being concentrated at specific sites within the oocyte. These findings provide additional evidence that orb function is required for the proper localization of orb mRNA. Moreover, as was the case for translation, Orb mediates localization through cis-acting sequences in the 3’ UTR of the orb message.

Orb protein associates with the 3’ UTR of orb mRNAs in vivo

The results described above suggest that Orb autoregulates both the translation and localization of orb mRNA through sequences in the 3’ UTR of the message. Since Orb is predicted to be an RNA binding protein, it could carry out these functions by interacting with orb mRNA. To test this possibility, RNA isolated from ovary extracts immunoprecipitated with Orb antibody was reverse transcribed with an ‘anchored’ oligo(dT) primer (see Materials and Methods) and the reverse transcription products were then PCR amplified using an upstream primer specific for the orb 3’ UTR and a downstream primer corresponding to the ‘anchor’ sequence. The PCR products were then detected with a 32P-labeled orb cDNA probe. To control for non-specific association of orb mRNA with antibody-bound beads, we reverse transcribed and PCR amplified (using the same primers) RNA immunoprecipitated from ovary extracts using Dorsal antibody. orb mRNA is absent in the Dorsal immunoprecipitates while it can be readily detected in the Orb immunoprecipitates (Fig. 6).

To assess the specificity of the orb mRNAs:Orb ribonucleoprotein complex, we RT-PCR amplified RNA isolated from the Orb and Dorsal immunoprecipitates using the downstream anchored primers and upstream primers for two localized mRNAs, bicoid and nanos, which are not orb regulatory targets (Christerson and McKearin, 1994). These mRNAs are not present in the Orb immunoprecipitates (Fig. 6). We also used a RT-PCR primer pair derived from the protein coding region of the orb message. In contrast, to the anchored oligo(dT) 3’ UTR primer pair, we did not detect orb amplification products with these upstream primers (data not shown). Since RNAs are partially hydrolyzed during the immunoprecipitation procedure, this finding suggests that Orb is complexed with sequences in the orb 3’ UTR.
Protein co-migrating with Orb can be cross-linked to orb mRNA

The Xenopus CPEB protein binds to a U rich sequence (UUUUAU, UUUUAAU and variants thereof) that is present in several masked mRNAs. Since Orb has RRM and zinc finger domains that are closely related to the CPEB protein, it would be reasonable to suppose that Orb recognizes similar RNA sequences. Inspection of the UTR reveals that there are approximately 16 U rich sequences, which resemble the binding sites for the Xenopus protein (see Fig. 7). Four of these have the consensus CPE sequence UUUUAU, while the remaining twelve are variants. Two of the UUUUAU sequences, and twelve of the variants are included in the 3'UTR of the HD construct. The other two consensus CPE sequences are in UTR sequences located just upstream of the HD construct in a region of the UTR present in both female and male mRNAs (see Lantz et al., 1992).

We used UV cross-linking to test if Orb protein in ovary extracts can interact with exogenous orb 3' UTR RNA. Ovarian proteins cross-linked to RNA were displayed by gel electrophoresis, blotted to nitrocellulose and visualized by autoradiography. At least 6 distinct 32P-labeled bands were detected (Fig. 7). Three of these, a 100 kDa band, a weak 70 kDa band, and the lower band in the 40 kDa doublet, correspond closely in size to known ovarian proteins, Orb, Bruno, and Squid respectively (Kelley, 1993; Kim-Ha et al., 1995; Lantz et al., 1994). Since nos mRNA is absent in Orb immunoprecipitates, we used a probe from the nos 3' UTR for UV cross-linking. As expected, the approx. 100 kDa band was not labeled by the nos probe, though other protein species (including the approx. 70 kDa and approx. 40 kDa bands) were observed (not shown).

To determine if the 100, 70 and 40 kDa 32P-labeled bands co-migrate with Orb, Bruno and Squid proteins, we first probed the nitrocellulose blot with Orb antibody. After visualizing Orb, we re-probed the same blot with Bruno antibody and subsequently Squid. In the Bruno re-probe, both Bruno and Orb are observed (Fig. 7), while all three proteins are observed in the Squid reprobe (not shown). We then overlayed the films to ascertain if the three bands in the western blot could be simultaneously aligned with the 32P-labeled protein species seen in the autoradiograph. As illustrated for Orb and Bruno in Fig. 7, we found that the 32P-labeled 100 kDa band co-migrates with the upper band of the Orb protein doublet, while the weaker 32P-labeled 70 kDa band co-migrates with Bruno. We also found that the lower band in the 40 kDa doublet co-migrates with Squid (not shown). We subdivided the orb 3' UTR into two smaller probes, B and C. Since each of these smaller probes is predicted to contain one of the consensus CPEB sites, plus 5 non-consensus sites, both would be
expected to cross-link Orb protein. Both smaller probes label protein species that co-migrate with Orb. While these results are consistent with the suggestion that the 32P-labeled, 100 kDa, band is Orb, we cannot exclude the possibility that some other, unknown protein of the same size as Orb binds to the orb transcript in our cross-linking experiments.

**DISCUSSION**

The RRM-type RNA binding protein Orb plays a central role in the establishment of polarity in the *Drosophila* egg and embryo (Lantz et al., 1994; Christerson and McKearin, 1994). In addition to its functions early in oogenesis in the formation and initial differentiation of the egg chamber, orb is required for the establishment of the DV and AP axes. In DV axis formation, Orb protein is required to localize grk mRNA to the dorsal anterior part of the oocyte and promote translation. In AP axis formation, the translation of osk mRNA localized at the posterior pole of the oocyte also depends upon Orb. Critical to its functions in these polarity pathways, Orb protein must be properly localized within the developing oocyte. In the studies reported here we have investigated the mechanisms directing the expression of Orb protein in the appropriate pattern. Our results suggest that autoregulation plays a critical role in promoting the on site accumulation of Orb protein. In this model, translationally repressed orb mRNA synthesized in nurse cells would be transported into and targeted to specific sites within the oocyte. Orb protein already present at these sites would bind to the orb mRNA when it arrives, anchoring the message to the cortex of the oocyte and activate its translation. Newly synthesized Orb protein would then be available to interact with incoming localized mRNAs and activate their translation. Once initiated, this orb autoregulatory activity would ensure the accumulation of high levels of Orb protein at sites in the oocyte containing localized orb mRNAs.

**Orb autoregulates its own expression**

The autoregulatory model was initially suggested by the dominant negative activity of transgenes expressing lacZ mRNAs which have the orb 3' UTR. As in classical antimorphic mutations, the phenotypic effects of the lacZ orb 3' UTR transgenes can be exacerbated by increasing the transgene dose relative to the endogenous orb gene. Conversely, it is possible to suppress the phenotypic effects of the orb 3' UTR transgenes by increasing the relative dosage of the endogenous gene. Since β-galactosidase has no adverse effects on orb function, the antimorphic activity of these transgenes can be attributed to the orb 3' UTR sequences in the transgene mRNAs. To have an antimorphic activity, these RNA sequences must interfere with the functioning of the endogenous gene. The most plausible mechanism is that the transgene 3' UTR sequences compete with mRNA from the endogenous gene for some limiting factor that is essential for the expression of sufficient quantities of Orb protein. Since orb is haploinsufficient, the obvious candidate for this limiting factor is the Orb protein itself.

One prediction of the autoregulatory model is that the expression of β-galactosidase from the lacZ orb 3' UTR mRNA should depend upon orb function. This is case. We found that Orb is required for the translation of the lacZ orb 3'UTR mRNA, and that the synthesis of β-galactosidase from the lacZ orb 3' UTR message is substantially reduced in orb mutant ovaries. In contrast, orb mutations have no effect on the translation of lacZ mRNAs that have unrelated 3' UTR sequences. A second prediction of the autoregulatory model is that the lacZ orb 3'UTR mRNA should compete with the endogenous message for orb function. Consistent with this prediction, increasing the relative dose of the transgene lacZ orb 3' UTR mRNA down regulates Orb protein expression from the endogenous gene. Taken together with the dominant negative activity of the lacZ orb3'UTR transgene evident in genetic assays these two lines of evidence provide strong support for the autoregulatory model.

Since Orb is an RRM type RNA binding protein, the simplest hypothesis is that Orb activates translation by interacting with orb mRNA rather than indirectly by controlling the synthesis or functioning of some other protein that binds to the 3' UTR. Two lines of evidence support the idea that Orb associates with orb mRNA in vivo. First, the defects in orb mRNA localization evident in orb mutant ovaries. Both the endogenous orb mRNA and the lacZ orb 3’ UTR transgene mRNA are not properly localized in the absence of wild-type orb activity. The abnormalities in localization, particularly the loss of the anterior ring along the nurse cell-oocyte margin, are consistent with a failure to properly anchor orb message to the oocyte cortex. Second, orb mRNA is associated with Orb protein in an immunoprecipitable complex in vivo. This complex appears to be specific as neither bcd nor nos can be detected in the immunoprecipitates. We have also assayed the Orb immunoprecipitates for 3’ UTR sequences from four mRNAs, osk, K(10), grk and Bic-D that exhibit defects in localization and translation in orb mutant ovaries (Lantz et al., 1994; Christerson and McKearin, 1994; unpublished data), and could potentially be targets for orb regulation in vivo. Of these four, osk, K(10) and Bic-D are present in Orb immunoprecipitates, while grk is not (Chang et al., 1999; L. Tan, unpublished data).

An obvious question is whether Orb binds directly to orb or any of the other mRNAs found in the immunoprecipitates. Orb homologs (the CPEB proteins) in other species have been shown to recognize a U-rich ‘CPE’ sequences in the 3’ UTRs of masked mRNAs (Fox et al., 1989; Paris and Richter, 1990; Hake and Richter, 1994; Gebauer and Richter, 1996; Hake et al., 1998). It is interesting to note that CPE-like sequences are found in the orb 3’ UTR and also in the UTRs of osk, K(10), and Bic-D. In contrast, CPE-like sequences are not found in grk nos or bcd mRNAs. Also favoring a direct interaction is the finding that a protein species which co-migrates with Orb can be UV cross-linked to 32P-labeled sequences from the orb 3’ UTR RNA. However, even if Orb recognizes the CPE-like sequences in the orb 3’UTR, we cannot rule out the possibility that autoregulation is nevertheless indirect, and, for example, depends on activating the expression of some other protein which also binds to orb mRNA.

**Mechanism of autoregulation**

Assuming that Orb plays a direct role in autoregulation, two different mechanisms could potentially account for the autoregulatory activity. In the first, efficient translation of orb...
mRNA would depend upon localization to the oocyte cortex and Orb protein would be required because it functions as an anchor. In this model, the defects in the expression of β-galactosidase from the lacZ orb 3′ UTR mRNAs in the absence of wild-type Orb would be explained by a failure in localization. Similarly, the antagonistic effects of the lacZ orb 3′ UTR transgenes would be explained by the displacement of the endogenous mRNA from the cortex by the transgene mRNA. Since lacZ mRNAs lacking the orb 3′ UTR are not localized in the oocyte, but are translated even in orb mutants, this postulated requirement for cortical association would have to be a special feature of mRNAs containing the orb 3′ UTR. For example, a translational repressor might be displaced from the orb 3′ UTR when the message is associated with the cortex.

In the second, Orb protein would not only anchor orb mRNA to the cortex, but also actively promote its translation. In this case, cortical localization would not in itself be sufficient for the translation of either the orb or lacZ orb 3′ UTR mRNAs. Arguing in favor of a more active role is the fact that the Orb homologs of Xenopus and other species, the CPEB proteins, function in the translational regulation of masked maternal mRNAs. These CPEB proteins are thought to bind to target sequences in the 3′ UTRs of masked maternal messages, initially helping to ensure that the mRNAs remain translationally silent (de Moor and Richter, 1999; Minshal et al., 1999). In response to an oocyte maturation signal, the proteins then activate translation of the masked mRNAs by promoting cytoplasmic polyadenylation (Fox et al., 1989; Paris et al., 1991; Hake and Richter, 1994; Stebbins-Boaz et al., 1996; Sheets et al., 1994, Sheets et al., 1995).

It would be reasonable to suppose that the regulatory activities of Orb resemble the CPEB proteins of other organisms. In this case Orb would positively autoregulate its own expression by activating the polyadenylation of localized orb mRNAs. However, experiments aimed at demonstrating this point have been inconclusive. Using the anchored-dT RT-PCR procedure of Salles et al. (Salles et al., 1994), we found this point have been inconclusive. Using the anchored-dT RT-PCR procedure of Salles et al. (Salles et al., 1994), we found this point have been inconclusive. Using the anchored-dT RT-PCR procedure of Salles et al. (Salles et al., 1994), we found.

The autoregulatory cycle

While a positive autoregulatory feedback loop would provide a mechanism for ensuring that Orb accumulates at sites of localized orb mRNA, a number of important questions remain. Some of these can be illustrated by comparing orb autoregulation with the autoregulatory cycle of the Sex-lethal gene (Sxl) (Cline and Meyer, 1996). For Sxl, autoregulation is crucial to its function as a binary switch gene, on in females and off in males. When the gene is on, Sxl proteins promote their own synthesis by directing the splicing of Sxl pre-mRNAs in the productive female pattern. When the gene is off and no Sxl proteins are present, splicing is in the default male pattern and the resulting mRNAs do not encode functional proteins. At this point it seems unlikely that orb autoregulation is used as an on/off switch; rather we suspect that it serves to augment the on site accumulation of Orb protein. In this view, orb mRNAs would be translated at a low level in the absence of Orb, while translation would be upregulated in its presence. Consistent with this suggestion we have found that β-galactosidase is expressed from the lacZ orb 3′ UTR transgene in both orb343 and orb303 ovaries even though these mutants have little or no functional Orb. (There is, however, an important caveat that the lacZ orb 3′ UTR mRNA does not have the long 5′ UTR (which contains several short Orfs) and consequently may not fully reproduce the translational regulation of orb mRNA.)

A second question relates to the initiation mechanism. In the case of Sxl, productive splicing can only occur in the presence of Sxl proteins. Consequently, activation of the autoregulatory cycle depends upon a special initiation pathway which bypasses this requirement (Cline and Meyer, 1996). If orb mRNAs can be translated at a reduced level in the absence of Orb protein, a special bypass mechanism would be unnecessary. However, there remains the problem of generating the proper spatial pattern of Orb protein accumulation within the egg chamber. For example, if the autoregulatory cycle is activated inappropriately in nurse cells, it would promote the accumulation of Orb protein in these cells instead of at the proper sites in the oocyte. One possibility would be to link autoregulation to Orb protein localization – for example, only Orb protein associated with the oocyte cortex would be able to activate orb mRNA translation. In this model, the binding of free Orb protein to orb mRNAs in transit in the nurse cells or in the oocyte would either have no effect on translation or (given the activities of CPEB proteins in other species) might actually repress translation.

Another issue is the mechanism that limits the positive autoregulatory feedback loop, preventing the over expression of Orb protein. In the case of Sxl, several factors appear to be responsible for limiting protein accumulation. One is the comparatively low level of Sxl transcription, while another is the instability of the Sxl protein (Bell et al., 1991). In addition, in females Sxl proteins negatively regulate their own translation by binding to target sites in the 3′ UTR of the Sxl mRNAs (Yanowitz et al., 1999). For orb, there must be mechanisms that turn off the autoregulatory cycle once sufficient quantities of Orb protein have been synthesized at particular sites. Since the localization of orb mRNA changes during oogenesis, one mechanism might be turnover (or re-localization) of orb mRNA. Another possibility is that high levels of Orb protein inhibit, instead of promote, translation. Finally, if positive autoregulation is coupled to the binding of Orb protein to the cortex, then the cycle might be inactivated once all Orb protein target sites are occupied. Further studies will be required to resolve these questions.

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